TITLE: Aerobic Biodegradation of DI-n-Butylphthalate by Pure and Mixed Bacterial Species

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AEROBIC BIODEGRADATION OF DI-n-BUTYL PHTHALATE BY PURE AND MIXED BACTERIAL SPECIES

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ABSTRACT

Di-n-butylphthalate (DBP), an organic ester, is primarily used as a plasticizer during the preparation of explosive mixtures. It is abundantly found in the wastewater generated from DBP production plants and munitions manufacturing facilities and at concentrations high enough to create a serious environmental problem. This compound is also listed as a priority pollutant by the USEPA. In this research, the aerobic biodegradation of DBP was studied using several *Pseudomonas* species as well as mixed bacterial cultures in a batch reactor under suspended growth conditions. The work presented in this paper is part of a broader investigation on the biodegradability of gun propellant components. Initial screening experiments, performed in shaker flasks at 30°C on a gyratory shaker rotating at 200 rpm, indicated that *Pseudomonas putida*, *Pseudomonas resinovorans*, and *Pseudomonas cepacia* can all degrade DBP as a sole carbon source. However, *Pseudomonas putida* exhibited higher degradation rates compared to the other two species. In addition to pure bacterial species, mixed cultures obtained from an activated sludge system were found to degrade DBP in the absence of any external carbon sources. A number of batch degradation experiments were performed using different initial concentrations of DBP in order to study the rates of degradation of the compound by *Pseudomonas putida* and activated sludge. The temperature was set at 30°C and the pH was maintained at approximately 7 during the course of the experiment. The higher rate of destruction observed during the kinetic studies suggest that biodegradation is a viable option for the treatment of DBP-containing wastewater.

INTRODUCTION

Di-n-butylphthalate ($C_{16}H_{22}O_4$) is primarily produced by the esterification of phthalic acid using n-butylalcohol in presence of acid or alkali as a catalyst. This compound is being widely used as a plasticizer for the preparation of explosive matrix of double base gun propellant. In addition, it is also being used as cosmetics chemical, diluent in polysulfide dental impression materials, safety glass, printing inks, and adhesives. Due to all these industrial activities, it is abundantly found in industrial as well as municipal wastewater. This compound is characterized as a possible teratogen.[1] The target organs are kidneys,

nerves, and male reproductive system. Thus USEPA listed this compound as a priority pollutant.[2] An LC_{50} value of 100 ppb - 1 ppm for larvae of grass shrimp is reported in literature.[3]

The compound is a colorless oily liquid with a vapor pressure of 0.1 mm of Hg at 115°C and a solubility of 11 mg/L to 4500 mg/L in water listed in different literature.[3, 4] The higher water solubility poses a serious threat to the environment.

Several technologies are available for treatment of DBP containing propellant formulations such as incineration, chemical treatment, and biological treatment. Incineration would generate some byproducts which could be even more dangerous than the parent compound unless it is done under controlled physical conditions. That would increase the cost of the overall process. Chemical treatment generally require other chemicals and sometimes it also generates other byproducts which have to be treated further. Bioremediation, overcomes these problems at a lower cost. The final products in this treatment process are generally carbon dioxide and water.

Bioremediation is nothing but the use of naturally present microorganisms which use the organic carbon as primary energy source in presence of other inorganic nutrients under aerobic and/or anaerobic environments. This technology has been proved to be cost effective and safe for treatment of several hazardous organic chemicals.

Microbial degradation of DBP was reported in the literature. Wang *et al.* (1995)[5] found five bacterial strains which can degrade DBP as a sole carbon source. The degradation time was varied from 40 hours to 220 hours for different strains to degrade 100 mg/L of DBP in the suspension. However, the degradation time was reduced when immobilized cells were used. Biotransformation of this compound *by Pseudomonas fluorescens* isolated from subsurface environment was demonstrated by Chauret *et al.* (1995).[6] The experiments were conducted under aerobic as well as anaerobic conditions. Complete mineralization time was varied from 20 to 70 days in their studies. Wang and Grady (1995)[7] explored the effects of biosorption and dissolution on the biodegradation of DBP. Biosorption isotherms for DBP by live and dead bacteria was evaluated by Wang and Grady (1994)[4]. Biosorption was significant in both live and dead bacteria, however, it was more in dead bacteria. Pogány *et al.* (1990)[8] studied metabolism of di-n-butyl phthalate in cell suspension cultures of tomatoes. They found degradation of DBP via a unidentified intermediate to benzoic acid.

In this research, biological treatment of DBP is explored using several pure bacterial species as well as mixed bacterial consortia obtained from an activated sludge plant.

MATERIALS AND METHODS

Analytical Methods

DBP Assay

An HPLC (Varian Instruments Co., Houston, TX) equipped with a tunable detector (Model No. 9065), an autosampler (Model No. 9095), and a pump (Model No. 9010) was used in conjunction with a C8 Beckman Ultrasphere column, C8, 5μ, 4.6 mm x 25 cm (Alltech Associates Inc., Deerfield, IL) to measure the concentration of DBP. A mobile phase containing 15% water and 85% methanol with a flow rate of 1 ml/min was used to elute DBP and detection was performed at 254 nm. A calibration curve was prepared for DBP upto 100 ppm of DBP concentration and found to be linear within this range. The minimum detection limit was about 1 ppm.

Prior to analysis, aqueous samples from the reactors were centrifuged for 10 minutes at 12,000 rpm in an ultracentrifuge (IEC Centra-M, International Equipment Co.) to separate the biomass. Then, 60 μl of each sample was injected into the HPLC unit via the auto-sampler.

Biomass Assay

The biomass concentration of the bacterial culture was determined by determining the optical density (OD) measurements. To determine the relationship between optical density and the actual biomass concentration, a calibration curve of optical density versus biomass concentration (mg/L) was prepared. Biomass concentration was determined by measuring the optical density at 540 nm by a diode array spectrophotometer (Hewlett Packard, Model No. 8452). Calibration curves were prepared for both *P. putida* and activated sludge. The procedure followed to develop the calibration curve is described below.

Respective bacterial culture was grown using glucose as the carbon source in a bioreactor. When, the glucose concentration was below detection limit, 100 ml of homogeneous solution was withdrawn from the reactor to determine the total suspended solid. At the same time, optical density of the same solution was measured. This procedure was followed for several dilution of the original liquor. Then the calibration curve was drawn between total suspended solid and optical density.

Organism and Inoculum

All pure cultures used in this study were obtained from the American Type Culture Collection (ATCC). The cultures were stored in a refrigerator at 4°C and transferred when needed. Mixed bacterial consortia or activated sludge was obtained from Linden POTW, Linden, New Jersey.

Growth Media

All growth media and solutions were prepared with deionized water unless mentioned otherwise. The basic composition of growth media was adopted from the ATCC manual. The growth medium used to grow bacteria and activated sludge in all experiments had the composition given in Table 1. The final pH of the solution was between 7.0 to 7.1

TABLE 1: Composition for bacterial growth media

Compound	Amount
Sodium phosphate (Dibasic)	11.2 g
Potassium Phosphate monobasic	5.7 g
Ammonium Sulfate	500 mg
Magnesium Sulfate	100 mg
Manganese Sulfate	10 mg
Ferric Chloride	5 mg
DI Water	1 liter

Experimental Procedures

A number of experiments were conducted to study the biodegradation of DBP. Initial screening experiments were conducted in shaker flasks. Later lab-scale bioreactors were used. The various set-ups and experimental procedures are described in this section.

Shaker Flask Experiment

The purpose of these experiments was to screen and identify various microorganisms to degrade DBP. All shaker flask experiments were conducted in 250 ml Erlenmeyer flasks with a liquid content 100 ml. The

mouths were closed with sterilized tissue paper. An incubator equipped with a gyratory shaker (Gallenkamp, Model # 905) was used for shaking the flasks at 100 rpm. The temperature in the shaker was maintained at 30°C for all the experiments conducted unless otherwise mentioned.

Bioreactor Experiments

A batch reactor (Bioflo II C, New Brunswick Scientific Co., NJ) with a 2.5 liter working volume was used to carry out a number of experiments for the biodegradation of DBP. The reactor was equipped with built in pH, dissolved oxygen and temperature sensors and control systems. The culture was acclimated in the presence of DBP in order to reduce the lag phase. Growth media solution containing certain amount of DBP was placed in the reactor, which was maintained at 30°C, and the solution was inoculated with pure culture or activated sludge. The reactor content was aerated at 2.0 l/min. The pH, DBP, and biomass concentrations were monitored and recorded at specified time intervals.

Desorption Experiments

After the end of each experiment, desorption experiment was conducted to verify any adsorption of DBP on the biomass. The experiment was conducted as follows: 40 ml of suspension was taken from the reactor in a 40 ml vial and centrifuged to separate biomass from the solution. Separated biomass was then mixed with 2 ml methanol and mixed thoroughly for an hour. The solution was then centrifuged again to separate biomass. The clear liquid was taken and injected into HPLC for DBP analysis.

RESULTS AND DISCUSSION

Three aerobic bacteria, namely, *Pseudomonas resinovorans*, *Pseudomonas putida*, and *Pseudomonas cepacia* were tested in order to determine which culture can degrade DBP faster. The results of the preliminary screening for degradation of DBP in shaker flask are shown in Figure 1. For comparison purpose the concentrations were normalized by dividing each concentration by their respective initial concentrations. From Figure 1, it is evident that all three Pseudomonas species could degrade DBP within 2 days. However, the degradation rate was faster in case of *P. putida.*

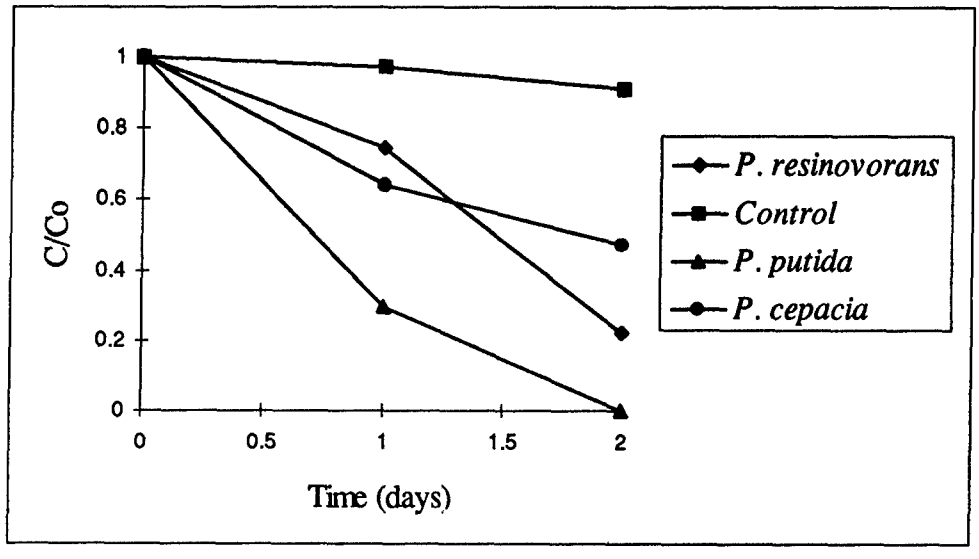

Figure 1. Degradation of DBP by Three Pseudomonas species for Screening Purpose

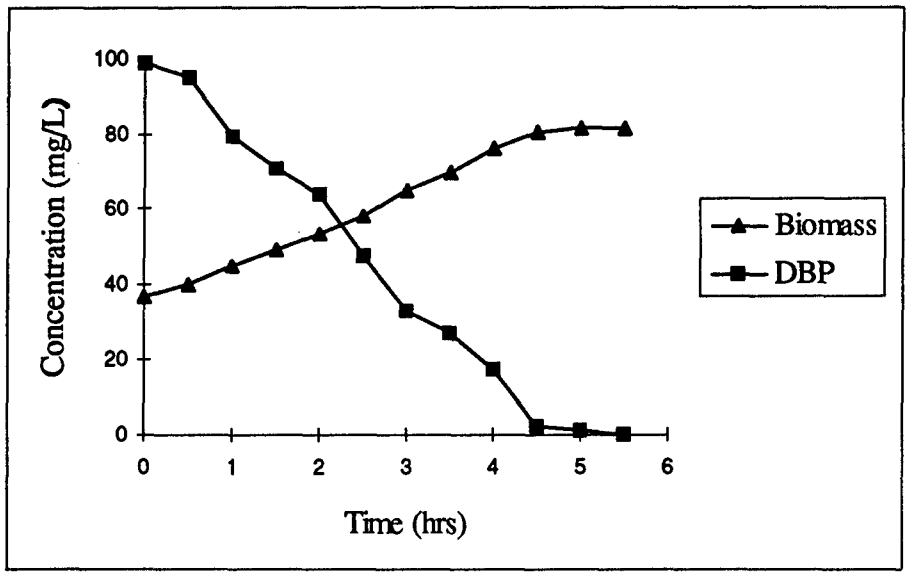

Figure 2. Degradation of DBP by *Pseudomonas putida*

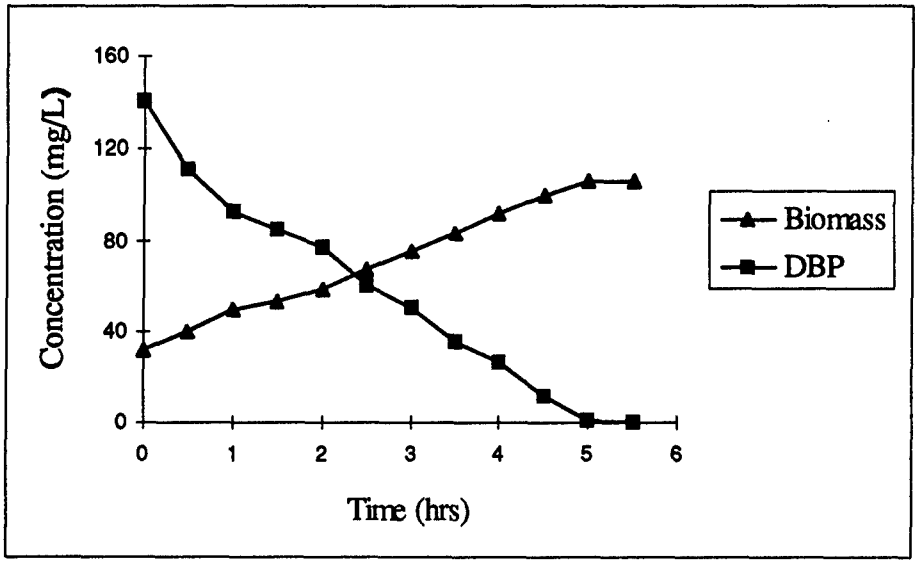

Figure 3. Degradation of DBP by mixed bacterial consortia.

Several experiments were conducted for the degradation of DBP by *P. putida* using different initial concentrations of DBP as well as biomass in a bench scale bioreactor. Initial DBP concentrations were ranged from 14 ppm to 140 ppm in 6 experiments. Typical experimental results are shown in Figure 2. Initial concentrations of DBP and biomass used in this experiment were 98 mg/L and 36 mg/L respectively. DBP and biomass concentration history are shown in Figure 2. It took only about 5.5 hrs to degrade DBP completely. The yield coefficient was about 0.46.

The next experiment was focused on the biodegradation of DBP by mixed microbial consortia in a bioreactor. Initial DBP and biomass concentrations were about 140 mg/L and 30 mg/L respectively. The results are shown in Figure 3. Within 5 hours, complete degradation was observed with yield coefficient of

0.53. The observed yield in this experiment was more than the observed yield by *P. putida* which indicates that mixed microbial consortia can tolerate higher doses of DBP without substantial inhibition.

Finally, the desorption experiment at the end of the degradation study showed that mixed bacterial species exhibited some adsorption of DBP on the biomass. However, with time the adsorbed amount of DBP on biomass decreased with time suggesting that biomass was able to degrade the adsorbed DBP.

CONCLUSIONS

The following conclusions can be drawn for the biodegradation of DBP.

- DBP is a chemical comparatively easy to degrade biologically and can serve as a sole carbon source for various pure and mixed bacterial cultures
- No inhibition was observed upto a concentration of around 140 mg/L for *P. putida* and mixed bacterial consortia.
- The rate of biodegradation by *P. putida* and mixed bacterial consortia were comparatively higher than other pure bacterial cultures.

ACKNOWLEDGMENTS

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